Abstract—We tested the ability of human mesenchymal stem cells (hMSCs) to deliver a biological pacemaker to the heart. hMSCs transfected with a cardiac pacemaker gene, mHCN2, by electroporation expressed high levels of Cs⁺-sensitive current (31.1±3.8 pA/pF at −150 mV) activating in the diastolic potential range with reversal potential of −37.5±1.0 mV, confirming the expressed current as Iᵢ-like. The expressed current responded to isoproterenol with an 11-mV positive shift in activation. Acetylcholine had no direct effect, but in the presence of isoproterenol, shifted activation 15 mV negative. Transfected hMSCs influenced beating rate in vitro when plated onto a localized region of a coverslip and overlaid with neonatal rat ventricular myocytes. The coculture beating rate was 93±16 bpm when hMSCs were transfected with control plasmid (expressing only EGFP) and 161±4 bpm when hMSCs were expressing both EGFP+mHCN2 (P<0.05). We next injected 10⁶ hMSCs transfected with either control plasmid or mHCN2 gene construct subepicardially in the canine left ventricular wall in situ. During sinus arrest, all control (EGFP) hearts had spontaneous rhythms (45±1 bpm, 2 of right-sided origin and 2 of left). In the EGFP+mHCN2 group, 5 of 6 animals developed spontaneous rhythms of left-sided origin (rate=61±5 bpm; P<0.05). Moreover, immunostaining of the injected regions demonstrated the presence of hMSCs forming gap junctions with adjacent myocytes. These findings demonstrate that genetically modified hMSCs can express functional HCN2 channels in vitro and in vivo, mimicking overexpression of HCN2 genes in cardiac myocytes, and represent a novel delivery system for pacemaker genes into the heart or other electrical syncytia. (Circ Res. 2004;94:952-959.)

Key Words: gene therapy ■ heart block ■ ion channels ■ pacemakers ■ stem cells

Although electronic pacemakers are currently the mainstay of therapy for heart block and other electrophysiological abnormalities, they are not optimal. Among their shortcomings are limited battery life, the need for permanent catheter implantation into the heart, and lack of response to autonomic neurohumors.¹ For these reasons, several gene therapy approaches have been explored as potential alternatives. These include either overexpression of β₂-adrenergic receptors,²-³ use of a dominant-negative construct to suppress inward rectifier current when expressed together with the wild-type gene Kir2.1,⁴ and implantation of vectors carrying the pacemaker gene, HCN2, into atrium⁵ or bundle branch system.⁶ A problem inherent in some of these approaches²-⁶ is the use of viruses to deliver the necessary genes. Although the vectors have been replication-deficient adenoviruses that have little infectious potential, these incorporate the possibility of only a transient improvement in pacemaker function as well as potential inflammatory responses. The use of retroviruses and other vectors, although not attempted as yet for biological pacemakers, carries a risk of carcinogenicity and infectivity that is unjustified, given the current success of electronic pacemakers. Attempts to use embryonic human stem cells to create pacemakers are still in their infancy and carry the problems of identifying appropriate cell lineages, the possibility of differentiation into lines other than pacemaker cells, and potential for neoplasia (see overview⁷).

With this in mind, we embarked on a project to test proof-of-principle that genetically engineered adult human mesenchymal stem cells (hMSCs) can serve as a platform for carrying the pacemaker gene to the heart. We did this with the understanding that the potential for differentiation into other cell lines exists for hMSCs as for embryonic stem cells, but with the rationale that if the relevant gene is genetically overexpressed then the presence or absence of differentiation

**Human Mesenchymal Stem Cells as a Gene Delivery System to Create Cardiac Pacemakers**


Integrative Physiology

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may be less important. Further, in testing proof-of-principle, we did not attempt to test the long-term safety of engineered hMSCs, which has yet to be demonstrated.

In this study, we demonstrate that hMSCs are effectively transfected by electroporation with a vector construct directing the expression of mouse HCN2 (mHCN2) as well as EGFP, and are capable of expressing functional mHCN2 channels in vitro. HCN2 expression in hMSCs provides an I_

f-based current sufficient to change the beating rate of cocultured neonatal rat ventricular myocytes, and to drive the canine ventricle, mimicking the HCN2 overexpression by adenoviral constructs. We demonstrate that hMSCs make connexin proteins and form functional gap junctions that couple electrically with canine cardiac myocytes. Thus, we have developed an ex vivo gene therapy system using genetically modified hMSCs as a platform for delivery of pacemaker genes into the heart.

Materials and Methods

Protocols were reviewed and approved by the Columbia University institutional animal care and use committee.

Human Mesenchymal Stem Cell Maintenance and Transfection

Human mesenchymal stem cells (Poietics hMSC; mesenchymal stem cells, human bone marrow) were purchased from Clonetics/BioWhittaker (Walkersville, Md) and cultured in MSC growing medium (Poietics MSCGM; BioWhittaker) at 37 °C in a humidified atmosphere of 5% CO₂. Cells were used from passages 2 to 4. A full-length mHCN2 cDNA was subcloned into a pIREs2-EGFP vector (BD Biosciences Clontech). Cells were transfected by electroporation using the Amaxa Biosystems Nucleofector (Amaxa) technology. Expression of EGFP after 24 to 48 hours revealed transfection efficiency of 30% to 45%.

Patch-Clamp Studies of I_

fHNC2 Expressed in hMSCs

We used whole-cell patch clamp to study membrane currents in control hMSCs and those transfected with mHCN2, the gene encoding the α-subunit of the pacemaker current, I_

f. Expressed I_

f (ie, I_

fHNC2) was measured under voltage-clamp by an Axopatch-1B (Axon Instruments) amplifier. Patch electrode resistance was 4 to 6 MΩ. Pipette solution was kept constant at 35 °C. The pipette solution included (in mmol/L) aspartate 130, KCl 5.4, NaOH 2.3, MgCl₂ 1, KCl 5.4, CaCl₂ 1.0, HEPES 5, and glucose 10; pH 7.4. Pipette solution included (in mmol/L) aspartic acid 130, KOH 146, NaCl 10, CaCl₂ 2, EGTA-KOH 5, Mg-ATP 2, and HEPES-KOH 10; pH 7.2. Recordings were conducted with an Axopatch 200 amplifier and PClamp 8 software (Axon Instruments). The perforated patch technique was used, and amphotericin B (400 µg/mL, Sigma) was added to the pipette solution.

In Vivo Studies in Canine Ventricles

Stem cells were prepared as above. Under sterile conditions, after sodium thiopental induction (17 mg/kg IV) and inhalational isoflurane (1.5 to 2.5%) anesthesia, 23- to 27-kg mongrel dogs (Team Associates, Dayville, Conn) were subjected to a pericardectomy. We injected 10⁶ hMSCs containing HCN2-GFP or GFP alone subepicardially in 0.6 mL of solution into the left ventricular anterior wall, approximately 2 mm deep to the epicardium via a 21-gauge needle. Animals recovered for 4 to 10 days, during which their cardiac rhythms were monitored. They then were anesthetized with isoflurane, as above. Both cervical vagal trunks were isolated, the chest opened, and ECGs monitored. Graded right and left vagal stimulation was performed via standard techniques to suppress sinus rhythm such that escape pacemaker function might occur. Tissues were then removed for histological study.

Histological Methods

Unless otherwise indicated, samples of heart tissue were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4 or 6 micrometers. Some formalin-fixed sections were stained in a routine fashion with hematoxylin and eosin (H&E). Monoclonal mouse antibodies (DakoCytomation) raised against the vimentin and human CD 44 were used applying an avidin-biotin-peroxidase method. Mouse antibodies (Zymed Laboratories Inc) were used. Tissues were treated to remove wax and rehydrated by exposure to xylene for 6 minutes with three rinses followed by similar exposures to 100%, 95%, 50% ethanol, deionized water, and PBS. The sections were then exposed to 30% hydrogen peroxide for 10 minutes and were again rinsed in PBS for 50 minutes. The rehydrated sections were exposed to a 0.01 mol/L citrate buffer, which was heated to a boil for 10 minutes and then allowed to cool to room temperature. Polyclonal antibodies raised against connexin 43 (Cx43; Zymed Laboratories Inc) were used.

Statistics

Results are presented as mean±SEM. Statistical significance was determined by Student’s t test for unpaired data. A value of P<0.05 was considered significant.
Results

Transfection of hMSCs With mHCN2 and Demonstration of Pacemaker Current

Nontransfected hMSCs demonstrated no significant time-dependent currents during hyperpolarizations (Figure 1A). MHCN2-transfected hMSCs expressed a large time-dependent inward current activating on hyperpolarizations up to \(-110 \pm 10\) mV and deactivating during the following step to \(-20\) mV (Figure 1B). Figure 1C shows the \(I_f\) activation curve constructed from tail currents recorded in mHCN2-transfected hMSCs (see inset for sample currents). We fit the data with a Boltzmann two-state model, which yielded a midpoint \(V_{50} = -91.8 \pm 0.9\) mV and a slope factor of \(8.8 \pm 0.5\) mV (n=9). \(I_f\) was fully activated around \(-140\) mV with an activation threshold of \(-60\) mV. Inset shows representative tail currents used to construct \(I_f\) activation curves. Voltage protocol was to hold at \(-30\) mV and hyperpolarize for 1.5 seconds to voltages between \(-40\) and \(-160\) mV in 10-mV increments followed by a 1.5-second voltage step to \(+20\) mV to record the tail currents.

Figure 1. Functional expression of \(I_f\) in hMSCs transfected with mHCN2 gene. \(I_f\) was expressed in hMSCs transfected with the mHCN2 gene (B) but not in nontransfected stem cells (A). C. Fit by the Boltzmann equation to the normalized tail currents of \(I_f\) gives a midpoint of \(-91.8 \pm 0.9\) mV and a slope of \(8.8 \pm 0.5\) mV (n=9). \(I_f\) was fully activated around \(-140\) mV with an activation threshold of \(-60\) mV. Inset shows representative tail currents used to construct \(I_f\) activation curves. Voltage protocol was to hold at \(-30\) mV and hyperpolarize for 1.5 seconds to voltages between \(-40\) and \(-160\) mV in 10-mV increments followed by a 1.5-second voltage step to \(+20\) mV to record the tail currents.

To confirm the expressed current was \(I_f\), we executed the experiments illustrated in Figure 2. The voltage protocol (see Figure 2 legend for description) allowed us to determine the reversal potential of \(-37.5 \pm 1.0\) mV (n=8). Given the extracellular \([K^+]\) of 5.4 mmol/L, this reversal potential is consistent with the mixed selectivity of the \(I_f\) channel to \([Na^+\) and \([K^+]\].\(^{17}\) We also tested the effect of \(Cs^+\) to block the expressed current. \(Cs^+\) (4 mmol/L) reversibly blocked the inward currents but had little effect on the outward deactivating tail currents, consistent with \(Cs^+\) blockade of \(I_f\).\(^{18}\) We constructed the fully activated \(I-V\) relationships for the \(I_f\)-like current in Figure 2D. The plot reinforces the two major observations from the raw data.

Figure 2. Effect of extracellular application of \(Cs^+\) and measurement of the reversal potential of \(I_f\). \(I_f\) was recorded before (A), during (B), and after (C) external addition of 4 mmol/L \(Cs^+\). D. Fully activated \(I-V\) relationship of \(I_f\) in the absence and presence of \(Cs^+\). Voltage protocol was to hold at \(-30\) mV and hyperpolarize to \(-150\) mV for 2 seconds followed by a 1.5-second depolarization to voltages between \(-150\) and \(+20\) mV to record the tail currents necessary to construct the fully activated current-voltage relation followed by a 0.5-second step to \(-10\) mV.

[Diagram of Figure 1 and Figure 2 not provided in text format]
I consistent with the known properties of Cs⁺. Dependence of activation of Iᵢ in hMSCs (Figures 3 and 4). Two-pulse protocol was initiated from a holding potential of −100 mV. First step was to −100 mV for 1.5 seconds followed by a second step to −150 mV for 1 second. Voltage was then stepped to +15 mV for 1 second to rapidly deactivate the current and then returned to the holding potential.

First, inward but not outward Iᵢ-like currents are blocked by Cs⁺, and second, the zero current indicates a mixed selectivity consistent with the known properties of Iᵢ. In a separate protocol Iᵢ density at −150 mV was 31.1 ± 3.8 pA/pF (n=17). Membrane capacity for the transfected hMSCs was 110.8 ± 9.0 pF (n=17).

Neurohumoral Regulation of Iᵢ<sub>HNCN2</sub>

A potential advantage of biological over electronic pacemakers is their hormonal regulation. We therefore examined the effects of β-adrenergic and muscarinic agonists on Iᵢ recorded in the hMSCs (Figures 3 and 4). Figures 3A and 3B demonstrate that the currents at −80 and −100 mV in isoproterenol are larger than those in control, whereas the currents in both conditions are almost equal at −160 mV. This voltage-dependent difference is expected for a shift in the activation curve (Figure 3D). The half activation voltage (V<sub>1/2</sub>) was −96±0.9 mV in control and −84.4±0.2 mV in isoproterenol (n=4, P<0.01). The slope factor was 10.9±0.5 mV in control and 11.0±0.2 mV in isoproterenol (P>0.05). Using a two-pulse protocol to illustrate the shift in activation, the time-dependent current in the presence of isoproterenol is larger in response to the first step than control and smaller in response to the second step (Figure 3C). This is consistent with an ISO-induced positive shift in Iᵢ activation. Acetylcholine had no direct effect on the time-dependent current (n=3), due either to the absence of muscarinic receptors or to a low basal level of cAMP that could not be further reduced by acetylcholine inhibition of adenylyl cyclase. We therefore further tested whether acetylcholine could reverse the actions of isoproterenol (Figure 4). Examination of the response to the step hyperpolarizations to −80 and −100 mV indicate that addition of acetylcholine reduces the membrane currents. However, they are almost identical at −160 mV (Figures 4A and 4B), consistent with a negative shift in activation induced by acetylcholine. Figure 4D shows the activation curves in isoproterenol and isoproterenol+acetylcholine. The V<sub>1/2</sub> were −91.3±1.1 mV for isoproterenol and −106.6±0.8 mV for isoproterenol+acetylcholine (n=3, P<0.05). The slope factors were 14.6±0.9 mV in isoproterenol and 11.1±0.9 mV (n=3, P<0.05). We also used a two-pulse protocol (Figure 4C). The response to the first voltage step is larger in isoproterenol than in isoproterenol+acetylcholine, whereas the reverse is true for the second step. This is again consistent with a negative shift in activation induced by addition of acetylcholine. These results demonstrate that the hMSCs transfected with mHCN2 should respond to β-adrenergic and muscarinic agonists.

mHCN2-Transfected hMSCs Modulation of Impulse Initiation by Cardiac Myocytes

Having expressed the pacemaker gene in hMSCs, we hypothesized that the mHCN2-transfected hMSCs could influence excitability of coupled heart cells. Maximum diastolic potential was −74±1 mV (n=5) in neonatal rat ventricular myocytes cocultured with EGFP expressing hMSCs and −67±2 mV (n=6) in myocytes cocultured with hMSCs expressing mHCN2 (P<0.05). Spontaneous rate was 93±16 bpm in the former group (n=5) and 161±4 bpm in the latter (n=6, P<0.05). The reduced maximum diastolic potential is consistent with the observed threshold potential of the ex-
pressed current in the mHCN2-transfected hMSCs, and indicates the influence of this depolarizing current on the electrically coupled myocytes. Representative action potentials are shown in Figure 5.

mHCN2-Transfected hMSCs as a Biological Pacemaker in Intact Canine Heart

Given the demonstration of functional coupling of mHCN2-expressing hMSCs to myocytes in vitro, we then injected them into canine heart in situ (see Materials and Methods) to test whether pacemaker function was demonstrable. During sinus arrest, escape pacemaker function can originate in the left or right ventricle, as occurred here, with two of four animals receiving hMSCs expressing EGFP alone developing left and two developing right ventricular escape rhythms. In contrast, five of six animals receiving hMSCs expressing EGFP+mHCN2 developed rhythms originating from and pace-mapped to the left ventricle at a site whose origin approximated that of the hMSC injection. Moreover, the idioventricular rates of these animals was 61±5 versus 45±1 bpm in animals receiving hMSCs expressing EGFP alone (P<0.05). A representative experiment is shown in Figure 6.

Hematoxylin and eosin stain of the site of hMSCs injection revealed normal cardiac myocytes and dense areas of basophilic infiltration adjacent to the needle track (Figure 7A). The hMSCs were easily identified by their size (10 to 20 μm in diameter), large hyperchromatic nuclei, and scanty, deeply basophilic cytoplasm with no matrix. Although the hMSCs had a characteristic appearance with H&E staining, they were more precisely identified by using immunohistochemical stains. The hMSCs stained intensely for vimentin (eg, Figure 7B), a marker of cells of mesenchymal origin. The same regions also were positive for human CD44 (eg, Figure 7C). Interdigitation between hMSCs and myocardium was very clear (eg, Figure 7D).

hMSCs Form Gap Junctions With Cardiac Myocytes In Vitro and In Vivo

To test whether the hMSCs couple electrically with cardiac myocytes, we cocultured hMSCs with adult canine ventricular myocytes. Myocytes were dissociated and plated for between 12 and 72 hours before coculture with hMSCs. Measurement of coupling occurred 6 to 12 hours after adding hMSCs to the myocyte culture. Our preliminary observations reveal that stem cells couple to cardiac cells. Figure 8A illustrates one example of an hMSC-myocyte pair in coculture; it is one of four so far observed. For heterologous pairs identification the hMSCs were tagged with Cell Tracker green (Molecular Probes). A bipolar voltage-ramp protocol was used to alter transjunctional voltage $V_j$ ($V_2-V_1$) over ±100 mV range at 200 mV/15-second rate (see $V_1$ and $V_2$) and is shown in Figure 8B. The ramp pulse was applied to the myocyte ($V_1$) while membrane potential of the hMSC was kept at 0 mV ($V_2$). The associated sister currents, $I_1$ and $I_2$, were recorded from the myocyte and hMSC, respectively.

Figure 5. Pacemaker function in in vitro model. Spontaneous electrical activity of neonatal rat ventricular myocytes cocultured for 4 to 5 days with hMSCs transfected with EGFP alone (A) or mHCN2 and EGFP (B). Experiments were conducted at 35°C.

Figure 6. Pacemaker function in canine heart in situ. Top to bottom, ECG leads I, II, III, AVR, AVL, and AVF. Left, Last two beats in sinus rhythm and onset of vagal stimulation (arrow) causing sinus arrest in a dog studied 7 days after implanting mHCN2-transfected hMSCs in LV anterior wall epicardium. Middle, During continued vagal stimulation, an idioventricular escape focus emerges, having a regular rhythm. Right, On cessation of vagal stimulation (arrow), there is a postvagal sinus tachycardia.
The currents followed the voltage-ramp profile demonstrating gap junction coupling of the heterologous hMSC-myocyte pair. The current, \( I_2 \), obtained from the nonstepped hMSC, reflects a coupling current, \( I_c \). This record demonstrates effective coupling of the hMSC to the ventricular myocyte. Figure 8C shows immunohistochemical staining with anti-Cx43 antibodies of the site of the injection of hMSCs into the canine heart. Intercalated discs are revealed in the mycardium (see purple arrow), whereas small punctate staining for Cx43 is seen between hMSCs (white arrows). There is also Cx43 staining at interfaces between hMSCs and myocytes (red arrows). The inset of Figure 8C shows a section from a piece of myocardium (fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer at pH of 7.4 at 4°C and subsequently treated as described by Walcott et al.19) injected with hMSCs expressing EGFP plus HCN2. The red staining from the secondary antibody to EGFP illustrates localization of hMSCs, whereas the blue staining illustrates cell nuclei. A significant majority of the clustered cells are hMSCs.

Discussion

Pacemaker implantation is a primary treatment for complete heart block or sinus node dysfunction. The current therapy uses electronic devices with high reliability and low morbidity. Nevertheless, such devices are not optimal because they lack the biological responsiveness of native tissues. Recently several approaches have been attempted to provide biological pacemaker function. Included among these attempts have been an upregulation of \( \beta_2 \)-adrenergic receptors, a downregulation of the background K\(^+\) current \( I_{K1} \), and our own previous studies with overexpression of the HCN2 gene, the molecular correlate of the endogenous cardiac pacemaker current \( I_f \).2–6 In these latter studies, we showed that HCN2 overexpression locally in left atrium or in the proximal bundle-branch system induces both \( I_f \)-like currents and in situ pacemaker function in the recipient myocytes. The unique voltage dependence of the \( I_f \) conductance results in current flow during diastole but not during the action potential plateau, limiting possible complications attendant to significant alterations of the action potential waveform. Although an adenoviral construct has been used to deliver the HCN2 gene to the heart,5,6 this approach is not optimal because adenoviruses are episomal and the nucleic acids they deliver do not integrate into genome. Other viral systems are accompanied by a number of serious drawbacks that hinder their use in vivo.

An alternative means for fabricating biological pacemakers is via embryonic stem cells, which can be differentiated along a cardiac lineage and might provide a platform for cell-based control of cardiac rhythm. Embryonic stem cells can make functional gap junctions and generate spontaneous electrical activity.20 However because of their immunogenicity, rejection is a serious consideration. Moreover, as with hMSCs, embryonic stem cell preparations are not spatially uniform and the proper engineering of both cell-based systems presents a challenge in designing in vivo biological pacemakers.

For several reasons, hMSCs are an attractive cellular vehicle for gene delivery applications. They can be obtained in relatively large numbers through a standard clinical procedure. hMSCs are easily expanded in culture and capable of long-term transgene expression.21 Their administration can be autologous or via banked stores, given evidence that they may be immunopriviliged.22 Long-term function of such a pacemaker is based on prolonged expression of mHCN2, which in turn requires integration into the genome of hMSCs. Random
The model system for this study. Our genetically engineered hMSCs expressed an $I_f$-like current and were capable of increasing the spontaneous beating rate of cocultured rat neonatal myocytes and originating a ventricular rhythm during vagally induced sinus arrest in the canine heart. Control hMSCs expressing only EGFP did not exert these effects either in vitro or in vivo. Thus, the electrical effects of the hMSCs transfected with the mHCN2 gene were similar to the effects of overexpression of the same gene in the myocytes in vivo and in vitro systems. These findings suggest that hMSCs may serve as an alternative approach for the delivery of pacemaker genes for cardiac implantation.

In sinus node myocytes the HCN gene generates an inward current necessary for cardiac excitation. Unlike sinoatrial node cells, mHCN2-transfected hMSCs are not excitable, because they lack the other currents required to generate an action potential. However, these cells are able to generate a depolarizing current, which spreads to coupled myocytes, driving myocytes to threshold. Our hypothesis is that as long as the hMSCs contain the pacemaker gene and couple to cardiac myocytes via gap junctions, they will function as a cardiac pacemaker in an analogous manner to the normal primary pacemaker the sinoatrial node. We demonstrated using dual patch technique that hMSCs form gap junctions that couple electrically with canine cardiac myocytes. The coupling between engrafted hMSCs and cardiac myocytes was also shown by immunohistochemical staining of the tissues isolated from the site of hMSC injection using anti-connexin 43 antibodies. Within an injection site, the clusters of cells were vimentin and CD44 positive, and we also demonstrated that a significant majority of the cluster of cells were EGFP positive, thereby confirming their identity as hMSCs. A recent report has suggested that mouse MSCs can fuse with mouse myocytes in vivo with a fusion rate of 0.005%. We have not ruled this possibility out in our studies, but at the fusion rate reported by Morimoto et al only 50 hMSCs of the million cells injected would fuse.

There are limitations to the approach used in this study. First, the hMSCs were delivered to the free wall myocardium, not an optimal site for ordered contraction. However, we have recently used catheter approaches to insert pacemaker genes into the canine left bundle branch system. Such a locus offers the possibility of more ordered and normal activation and contraction than is the case with a pacemaker residing in the free wall. Before this approach is used for hMSCs, catheter modification may need to occur to optimize injection of cells of the size of an hMSC without cell injury or destruction.

Another question relates to the duration of efficacy of these pacemakers. In the present study, we were only concerned with demonstrating the feasibility of using hMSCs as a gene delivery system. Because our studies in vivo lasted only 3 to 10 days, transient transfections were sufficient. Before this approach can be considered clinically relevant, far longer periods of study will be required. In this regard, our transfected cells maintain their green fluorescence for at least 3 months when grown on antibiotic to select stably expressing cells. This indicates that we have selected for stable clones expressing mHCN2, so it is likely that persistence of expression will not pose significant difficulties for more prolonged...
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